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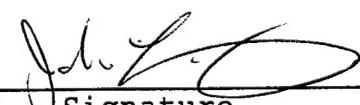
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13. ABSTRACT (Maximum 200) Development of breast cancer is a multistep process involving a complex interplay of both inherited and acquired genetic alterations. In this grant, we have proposed to pursue several lines of studies to define the role of the met tyrosine kinase in the development of breast cancer. First we plan to characterize the structural and functional alterations of the met gene and its product(s) in breast cancers. Second we wish to define the molecular basis of altered met expression in the pathogenesis of mammary epithelial neoplasia. Third we plan to assess the biological consequences of altered met expression with respect to the development and oncogenesis of breast tissues in animal model systems. In this progress report, we have evidence that c-met expression is altered in human breast cancer lines and that activated met oncogene can induce breast cancer development in transgenic mouse model.					
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FOREWORD

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J. L. S.

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I. Introduction

Breast cancer is the most common malignancy affecting women in the Western World. Epidemiological studies have defined certain factors that may contribute to the risk of breast cancer, the most important one being a family history of the disease (1, 2). Approximately 5-10% of breast cancers are associated with inherited susceptibility with one or more autosomal dominant traits. Linkage at the estrogen receptor on chromosome 6 has also been reported in breast cancer family with a late-onset mode (3). Families with germ-line p53 mutations (Li-Fraumeni Syndrome) often have multiple breast cancers, representing only about 1% of all familial breast cancer cases (4). Molecular analysis of sporadic breast cancer DNAs has revealed loss of heterozygosity on chromosomes 1p, 1q, 3p, 6q, 7q, 8q, 11p, 13q, 15q, 16q, 17p, 17q, and 18q (5). Gene amplification is also frequently observed in the c-myc, HER2/neu, and int-2/PRAD-1 genes of breast tumors (6-8). Furthermore, a significant association between the level of nm23 expression and aggressive tumor behavior has been demonstrated (9). Recently, a breast cancer susceptibility gene (BRAC1) in chromosome 17q13 has been identified (10). These observations suggest that breast cancer, similar to the oncogenesis of other solid tumors, develops through a multistep process involving various genetic alterations. Identification and characterization of these genetic alterations will not only offer the possibility of early diagnosis but also spawn new treatment modalities targeted specifically at the altered phenotype of malignant cells.

Tyrosine kinases have been by far the most important class of mutated cellular genes during malignant transformation. The met oncogene was identified in a N-methyl-N'-nitro-N-nitrosoguanidine(MNNG)-treated human osteosarcoma cell line HOS, using the NIH 3T3 cell transfection system (11-13). The activation of the met oncogene was shown to occur via a chromosomal rearrangement, presumably as a result of the mutagenic effect of MNNG. The rearrangement generates a chimeric gene, fusing an upstream promoter-containing sequence (tpr) from chromosome 1 in front of the carboxyl terminus of the met protooncogene on chromosome 7. The fusion molecule (MW 65 KD) contains the tyrosine kinase domain of the met protooncogene. The tpr sequence consists of a constitutive promoter and an open reading frame coding for a protein with strong sequence homology to nuclear oncoproteins fos, jun, transcription factor CREB, and members of intermediate filament multigene family (14). The common feature

among these molecules is that they contain a leucine zipper which has been shown to be required for dimerization and activation of these proteins (15). Recent data demonstrated that tpr-met oncogene was indeed activated through this leucine zipper interaction, resulting in a constitutively phosphorylated and presumably active state of this tyrosine kinase molecule (16). Overexpression of normal c-met appears to be sufficient to activate the tyrosine kinase, which may explain the transforming potential of amplified c-met gene in some human tumors (17). The identification and characterization of other forms of c-met has demonstrated that abnormal processing of the extracellular domain of the protein can also result in constitutive activation of c-met (17). Similar to what has been described for other receptor tyrosine kinases (RTK), such as trk and ret proto-oncogenes, mutations affecting the extracellular or transmembrane domain may be the molecular basis for the oncogenic potential of met in some human cancers (18-21).

The proto-oncogene of met encodes a receptor of 190 kDa protein, composed of two disulfide-linked subunits: an extracellular 50 kDa α -subunit and a transmembrane 145 kDa β -subunit. The receptor is synthesized as a 170 kDa precursor that is glycosylated and cleaved posttranslationally to give the mature heterodimer. The intracellular domain of c-met protein has a structure resembling that of the protein tyrosine kinase (PTK) family. Recent studies have shown the presence of multiple forms of c-met gene products, presumably as a result of alternative splicing (22). The functional significance of these alternative forms has remained largely unknown.

Recently, hepatocyte growth factor (HGF) has been shown to be the ligand for the c-met receptor (23). HGF is also known as hepatopoietin and is identical to scatter factor, which affects the motility, chemotaxis, and invasiveness of epithelial and endothelial cells in culture (24-26). HGF has been shown to be the most potent growth factor for rat and human hepatocytes in primary cultures (27). In addition to its mitogenic effect, it regulates cellular shape as a morphogen and cellular motility as a motogen (24). The pleiotropic effects of HGF-SF on cells suggest a complex interplay of receptor-mediated signal transductions. It is clear from previous studies that HGF appears to induce rather diverse biological effects in various cell types (24). The factors determining the outcome of HGF actions have remained largely unknown. Elucidation of these factors will undoubtedly give us some unique insights into the

molecular mechanisms of growth regulation and malignant transformation.

Cytogenetic studies of transformed cells derived from human malignancies suggested that the mechanism of chromosomal rearrangement resulting in fusion gene products with transforming potential may be a common mechanism of oncogenesis (25). Several examples are the Philadelphia chromosome of chronic myelogenous leukemia, t(14;18) translocation of follicular B cell lymphoma, and t(10;14) translocation of T cell acute lymphoblastic leukemia. Therefore, activation of met proto-oncogene via this mechanism may be more than an in vitro observation in the original mutagenesis experiment described above. In addition, c-met proto-oncogene has been shown to be amplified and overexpressed in a human gastric tumor cell line, although the mechanism of activation is distinct from that of tpr-met activation (26). A recent report described the finding of similar chromosomal rearrangements resulting in tpr-met fusion gene in human gastric tumors (27). This provocative finding is consistent with the observation that nitroso compounds are epidemiologically associated with the occurrence of gastric cancer in humans (28).

Regardless of the oncogenic potential of the activated met protein, its proto-oncogene product likely plays an important role in the growth and differentiation of epithelial cells in various organs. Recent studies have demonstrated a wide tissue distribution of this protein, such as in breast, intestine, stomach, liver, pancreas, kidney, etc., and more interestingly, increased level of met RNA and protein in several carcinoma specimens, particularly in thyroid, gastric and intestinal tumors (29). In contrast, none of 15 primary breast cancers showed expression of met protein, whereas significant met expression was detected in 4 of 4 normal mammary epithelium (29). Another provocative study demonstrated that the c-met locus on chromosome 7 (7q21-22) was deleted in 41% of 245 patients with primary breast cancer (30). In addition, patients with loss of heterozygosity on chromosome 7q21-22 had significantly shorter metastasis-free survival and overall survival. Recent studies on the functions of HGF-met activation have suggested a potential regulatory role for c-met in the morphogenesis of breast tissues (31). These observations, *in toto*, suggest that this region of chromosome 7, possibly the c-met gene, may be the site of a breast tumor or metastasis suppressor gene. The concept of a RTK as a tumor suppressor gene has been substantiated by a recent report demonstrating that the RET tyrosine kinase gene appears to

code for the tumor susceptibility gene in Multiple Endocrine Neoplasia Type II Syndrome (MEN II) (32).

In this grant application, we plan to search for genetic abnormalities affecting the c-met gene at the DNA, RNA, and protein levels in human breast cancer cell lines and tumor specimens (Specific Aim A), and study the key cellular targets of c-met gene product in mammary epithelial cells and define the functional effects of mutated met proteins through exploring a innovative technology to identify and characterize interacting proteins (Specific Aim B). We are applying the two-hybrid system in yeast in order to clone cellular genes whose protein products interact specifically with the met protein. As Specific Aim C, we plan to develop animals models to examine the oncogenic potentials of met oncogene and to assess the role of met proto-oncogene in the growth and development of breast tissues.

II. Body

We are applying a variety of molecular and cellular approaches to study the role of c-met in the development of breast cancer. We have generated significant amounts of preliminary data regarding each of the specific aims. For Specific Aim A, we have examined the status of c-met gene and its product(s) in several breast cancer cell lines and have observed that the expression of met gene in these lines appeared to be absent or altered with aberrant forms of gene product (Table 1). For Specific Aim B, we have initiated our effort to clone cellular target(s) of c-met in order to understand the signal transduction pathway leading to malignant transformation of mammary epithelium using the two-hybrid system. This innovative technology has been developed and used extensively to clone and identify cellular factors interacting with specific proteins of interest. The system has been described in detail (33, 34).

In brief, two different DNA constructs capable of replicating in yeast are prepared (Fig. 1). In the first construct, the gene coding for the protein of interest is fused to a gene encoding a known DNA-binding polypeptide that dose not activate transcription by itself, such as the DNA binding domain of GAL4 (aa 1-147) or LexA (aa 1-202). The second construct containing a gene coding for an activation domain, such as that of GAL4 (aa 768-881) or other transcription activator, is fused to a cDNA library of interest, as the "prey." The second construct, by itself, does not activate transcription, since it contains no DNA binding domain.

The reporter construct contains the cognate DNA binding sequence of the DNA binding domain upstream of a gene that produces an protein with enzymatic activity, such as b-galactosidase (b-gal), or an auxotrophic marker, such as Leu-2, which is required for leucine synthesis. The interaction of two proteins will bring the DNA binding and activation domains together to activate the transcription of the reporter gene, resulting in selection of the yeast clone containing the candidate gene whose gene product interacts with the protein of interest. The system we have adopted was provided generously by Dr. Roger Brent of the Dept of Molecular Biology at MGH. We have identified several candidate genes whose gene products interact specifically with the intracellular domain of c-met in the yeast two-hybrid system (Table 2). Further characterization of these clones, hopefully, will lead to a better understanding of how mutated c-met induces breast cancers.

Finally for Specific Aim C, we have generated four transgenic founder lines expressing the met oncogene (tpr-met). Three lines positive for tpr-met transgene were successfully bred and established. The results of these studies have been published in the Journal of Clinical Investigation (see attached manuscript). In summary, A F1 female animal from the MTM1 line, after breeding for 6 months, developed a mammary cancer. Nine additional F1 females from this line, 8 from line MTM2, and 8 from line MTM3 were set up for forced continuous breeding (see attached manuscript). Most of the multiparous mice had mammary hyperplastic alveolar nodules (HAN) (see attached manuscript) and several of them also had foci of microscopic carcinoma on whole mount examination. Seven primary mammary tumors developed in 6 of the MTM1 animals. In the MTM2 line, 7 independent mammary tumors developed in three female animals. In the third line (MTM3), 3 mammary tumors were observed in two animals. The mammary tumors had one of three patterns, scirrhous, papillary or nodular , many of them resembling human mammary tumors. The nuclei were intermediate in size with delicate clumped chromatin. Histological patterns of these types are not seen in spontaneous murine tumors.

Additional tumors were also found in animals from all three lines. Single animals from MTM1 and MTM3 lines developed diffuse lymphoblastic lymphomas involving the mammary gland and lymph nodes at 6-9 months of age. One MTM3 mouse developed a thymic lymphoma. Spindle cell sarcomas were also observed in MTM2 and MTM1 mice. An unusual orbital giant cell osteosarcoma was noted in another MTM1 animal at 18 months of age. In addition to continuing

our analyses of these lines, we had hope to extend our efforts to study the biological effects of inactivated met gene in transgenic mice with germ-line disruption of the c-met gene in our original proposal. Unfortunately, the same study has been published recently in Science (35). Since the homozygous knock-out is embryonically lethal, it is difficult to analyze the effect of this genotype on mammary development. We are currently contemplating an alternative approach to address this issue. Techniques are now available for tissue-specific conditional knock-out and we plan to pursue this avenue of research.

As an overall goal of the proposal, by exploring these three distinct but complementary approaches, we hope to gain important insights into the multistep pathogenesis of breast cancer.

III. Conclusions

From our preliminary studies on genetic alterations involving the c-met gene, we observed an association with tumorigenicity of cell lines and aberrant expression of c-met (Specific Aim A). This observation is consistent with our hypothesis that dysregulated synthesis of c-met plays a role in breast carcinogenesis. Identification and characterization of signal transduction pathway of c-met in mammary tissues will likely provide us with important information regarding the molecular mechanism of met-induced mammary carcinogenesis (Specific Aim B).

We have performed extensive studies in the transgenic model of met (Specific Aim C) and these studies have already been published (attached manuscript). The pattern and occurrence of mammary hyperplasia and tumors in the tpr-met transgenic strains support strongly the conclusion that these lesions are a direct effect of the transgene. Hyperplasia and tumors developed in all three independent transgenic lines (MTM1, MTM2, and MTM3). In addition, no spontaneous mammary tumors developed in any of the nontransgenic littermates maintained in parallel. This observation is consistent with other investigator's experiences with the FVB strain. Furthermore, occurrence of multiple independent mammary tumors, as observed in some of our animals, is rare in spontaneous breast tumors of normal mice. The use of a cellular promoter (metallothionein) rather than the MMTV promoter to drive the tpr-met transgene further speaks for the uniqueness of this animal model in the study of mammary carcinogenesis. It is interesting to note that only breast tumors expressed high-level of the tpr-met transcript and protein, whereas normal tissues including mammary

epithelium expressed very little. Perhaps, during physiological hyperplasia of mammary glands as a result of pregnancy, cells expressing higher levels of the oncoprotein were selected and clonally expanded to eventually form foci of mammary malignancy. This observation suggests that mammary adenocarcinoma developed in this transgenic model as a direct effect of high-level expression of tpr-met.

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V. Appendix

Table 1: Altered c-met Expression in Breast Cell Lines.

Cell Line*	Tumorigenic**	DNA***	RNA#	Protein
75N	-	+	+	ND
70N	-	+	+	+
81NN	-	+	+	ND
21NT	-	+	+	+
21PT	-	+	+	ND
18-2-1	-	+	+	ND
1436N1	-	+	+	ND
HBL-100	-	+	+	+
Hs578Bst	-	+	+	+
MDA-MB-157	+	+	-	ND
MDA-MB-231	+	+	-	ND
MDA-MB-361	+	+	-	ND
MDA-MB-435	+	+	+	+
MDA-MB-436	+	+	+	ND
MDA-MB-468	+	+	-	ND
BT-474	+	+	-	ND
BT-549	+	+	-	-
MCF7	+	+	-	-
MCF10A	+	+	+	ND
T-47D	+	+	-	ND
ZR-75-1	+	+	-	-
ZR-75-3	+	+	-	-
DU4475	+	+	-	-
Hs578T	+	+	+	-
SK-BR3	+	+	+	-

* The primary mammary cells 76N and 70N, and immortalized breast cell lines 21-NT, 21-PT, 18-2-1, and 1436N1 are from Ruth Sager; the remaining cell lines are obtained thru ATCC.

** "Nontumorigenic" is defined as inability to form tumor in nude mouse.

*** Absence of gross gene rearrangement by restriction and Southern blot analysis is defined as "+".

presence or absence of met mRNA was indicated by + or -.

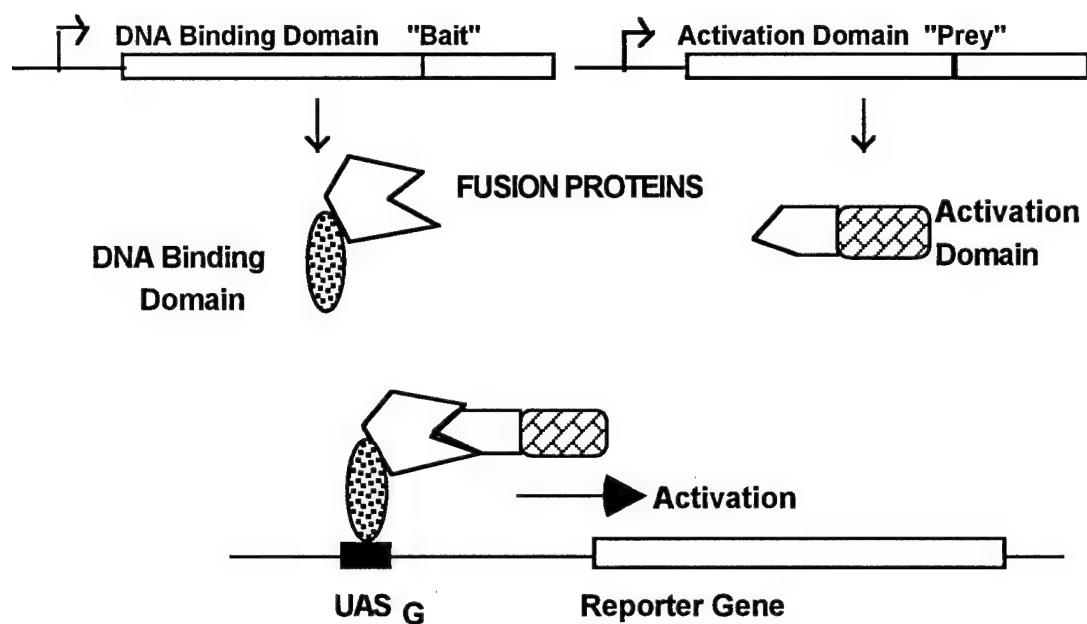
ND: Not done

Table 2. Interaction of Intracellular Domain of c--met with Candidate cDNA Clones

Yeast Plasmid Construct	β -gal Activity (U)	
	Glucose	Galactose
LexA-MetCD + JG4-5	10	12
LexA-MetCD + JG4-5-MAP51	25	1939
LexA-MetCD + JG4-5-MAP142	26	2789
LexA-MetCD + JG4-5-MAP191	31	1897
LexA-MetCD + JG4-5-MAP282	27	2192
LexA + JG4-5-MAP51	17	19
LexA + JG4-5-MAP142	15	21
LexA + JG4-5-MAP191	11	14
LexA + JG4-5-PMAP282	13	16

Figure 1.

The Yeast Two-Hybrid System



Transgenic Expression of tpr-met Oncogene Leads to Development of Mammary Hyperplasia and Tumors

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Abstract

Receptor tyrosine kinases are important in cell signal transduction and proliferation. Abnormal expression of tyrosine kinases often leads to malignant transformation. c-met is a tyrosine kinase receptor and its ligand is hepatocyte growth factor (HGF). HGF/c-met plays diverse roles in regulation of cell growth, shape and movement. Constitutively activated met, such as tpr-met, is a potent oncogene in vitro, but its carcinogenic role in vivo remains unclear. Our study demonstrates that expression of tpr-met leads to development of mammary tumors and other malignancies in transgenic mice, and suggests that deregulated met expression may be involved in mammary carcinogenesis. (*J. Clin. Invest.* 1996; 97:2872–2877.) Key words: c-met • hepatocyte growth factor • receptor tyrosine kinase • carcinogenesis • sarcoma

Introduction

The c-met protooncogene encodes a tyrosine kinase receptor of 190-kD protein. Hepatocyte growth factor (HGF)¹ is the ligand of the c-met receptor (1). HGF is also known as hepatopoietin and is identical to scatter factor, which affects the motility, chemotaxis, and invasiveness of epithelial and endothelial cells in culture (2–4). HGF has been shown to be the most potent growth factor for rat and human hepatocytes in primary cultures (5, 6). In addition to its mitogenic effect, HGF regulates cellular shape as a morphogen and cellular motility as a motogen (3). Recently, met-HGF/SF activation was also shown to mediate mesenchymal to epithelial transition (7).

The oncogenic form of c-met (tpr-met) was identified in a N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-treated human osteosarcoma cell line HOS (8–10). The activation of the met oncogene was shown to occur via a chromosomal rear-

rangement, generating a chimeric gene, fusing an upstream promoter-containing sequence (tpr) from chromosome 1 to the carboxyl terminus of the met protooncogene on chromosome 7. The fusion molecule (65 kD) contains the tyrosine kinase domain of the c-met protooncogene. The tpr sequence consists of a constitutive promoter and an open reading frame coding for a protein with strong sequence homology to nuclear oncoproteins fos and jun, transcription factor CREB, and members of intermediate filament multigene family (11). The common feature among these molecules is that they contain a leucine zipper which is required for dimerization and activation of these proteins (12). Recent data demonstrated that tpr-met oncogene was indeed activated through this leucine zipper interaction, resulting in a constitutively phosphorylated and presumably active state of this tyrosine kinase molecule (13).

Overexpression of normal c-met appears to be sufficient to activate tyrosine kinase activity, which may explain the transforming potential of amplified c-met gene in some human tumors (14). Met is frequently amplified and overexpressed in various transformed cell lines and human tumors (15, 16). The identification and characterization of other forms of c-met demonstrated that abnormal processing of the extracellular domain of the protein can also result in constitutive activation of c-met (14). Similar to what has been described for other receptor tyrosine kinases, such as trk and ret protooncogenes (17–19), activating mutations affecting the extracellular or transmembrane domain of c-met may contribute to an oncogenic potential of met in some human cancers. To date, no direct evidence exists demonstrating the oncogenic potential of met in vivo. Therefore, in order to assess met as a candidate oncogene in vivo, we examined the consequences of met oncogene expression in transgenic mice.

Methods

Transgenic construction. The 2.2-kbp cDNA clone of the tpr-met oncogene (9) was inserted into an expression construct containing the 1-kb mouse metallothionein 1 (MT1) promoter (20) and the SV40 small T intron and polyadenylation signal. The MT-tpr-met construct was transfected into COS cells and expression of the tpr-met protein was demonstrated by Western blot analysis (not shown). A linearized fragment from the MT-tpr-met construct was injected into fertilized mouse (FVB/n X FVB/n) eggs following established protocols. 33 potential founder pups were screened for transgene incorporation by Southern blot analysis. Four founder lines positive for the transgene were identified (MTM1-4). Three founders transmitted the transgene to their progeny in a Mendelian fashion, while the fourth line appeared to be a mosaic.

Analysis of tpr-met transgene expression. RNAs were extracted from various tissues using RNAzol (Biotecx Laboratories, Inc., Hous-

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1. Abbreviation used in this paper: HGF, hepatocyte growth factor.

Table I. Tumor Incidence in MTM Transgenic Strains

Transgenic line	Mice in continuous breeding	Mice with mammary tumors*	Independent mammary tumors	Mean age of onset [†]	Mice with other tumors [§]
MTM1*	10	6	7	317	3
MTM2	8	3	7	394	1
MTM3	8	2	3	433	2

Cohorts of mice in each of the MTM transgenic strains were observed for tumor development. Mammary and other tumors were observed in all strains. *Parallel cohort of nontransgenic litter mates (15) were set up for continuous mating as controls and none of the mice developed tumors at one and half years of age. [†]Data reported as mean age in days of detection of breast tumors. [§]These animals were not included in the cohort of female mice in continuous breeding. One female MTM1 animal developed diffuse lymphoma at 9 mo of age and a male had metastatic spindle cell sarcoma at two years of age. An orbital osteosarcoma was noted in the third male MTM1 animal at 18 mo of age. One MTM2 male mouse was noted to have two lesions: one squamous papilloma and the other rectal spindle cell sarcoma. Two female MTM3 animals developed lymphoma, one thymic and the other diffuse at an early age of 3 mo.

ton, TX), subjected to Northern blot analysis, and hybridized with ³²P-labeled human c-met cDNA under stringent condition. RT-PCR was performed using an antisense primer from the SV40 polyadenylation sequence (5' CCTGAAATGAGCCTTGGGA 3') as the RT primer and a sense primer from the COOH terminus of the tpr-met sequence (5' TCTTGCTCCTGCCATAGG 3') as the second primer. Control reactions without RT were performed in parallel for each samples. For analysis of protein, tissues were homogenized using a Dounce homogenizer in the presence of RIPA buffer containing protease inhibitors. For analysis of protein phosphorylation, phosphatase inhibitors (100 mM NaF, 20 mM disodium p-nitrophenyl phosphate, 10 mM disodium-bis-glycerophosphate, 10 mM tetrasodium pyrophosphate, 1 mM sodium othovanadate) were also included in the lysis buffer. Cell lysates were cleared of nuclei and other cell debris by centrifugation and subjected to SDS-PAGE (reducing condition) and Western blot analysis with anti-met 19 S monoclonal antibody (from George Vande Woude) in 1:1000 dilution using a chemiluminescence kit (Amersham).

Results

Three lines positive for tpr-met transgene were successfully established. An F1 female animal from the MTM1 line, after breeding for 6 mo, developed a mammary cancer. Nine addi-

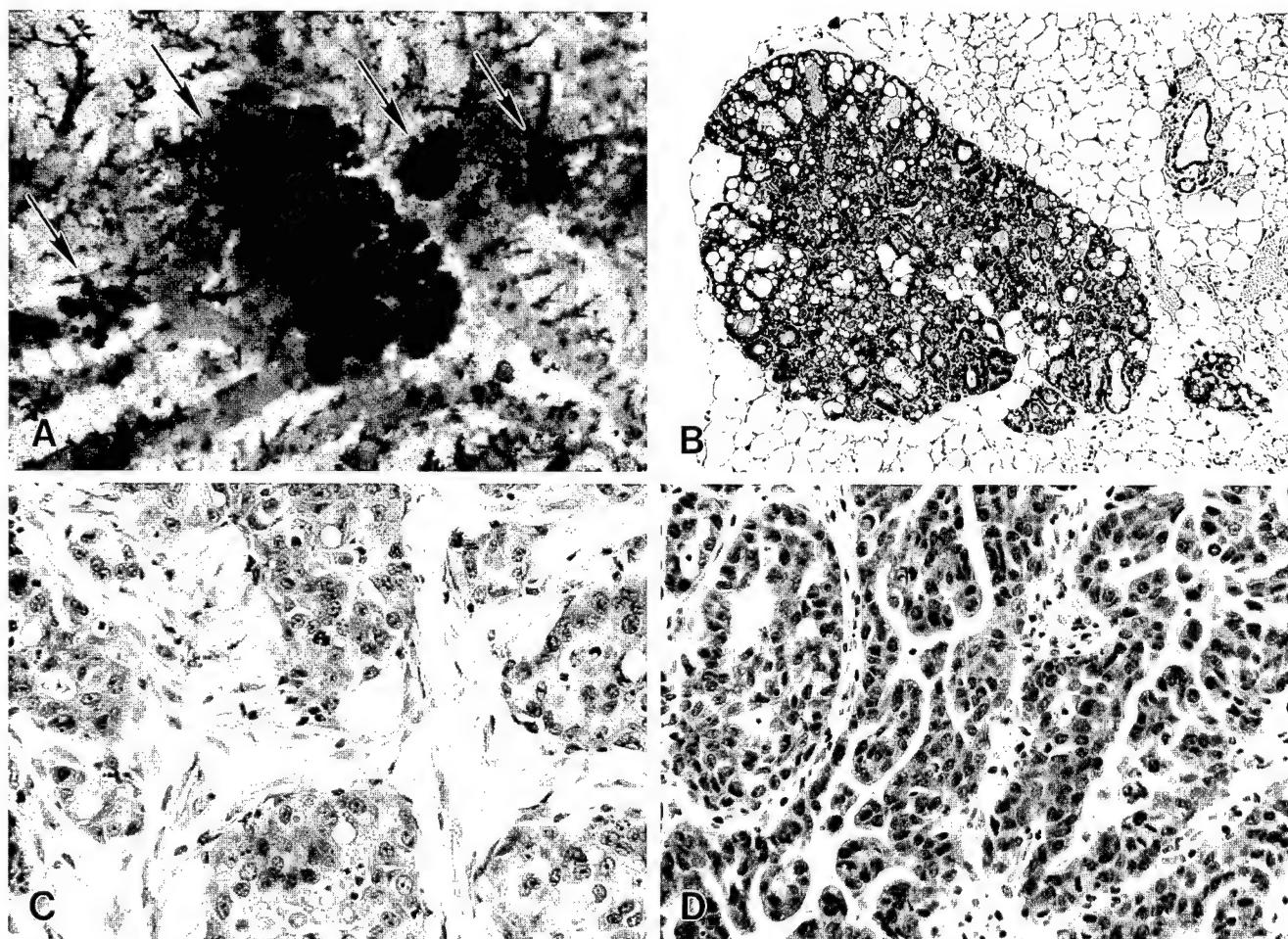


Figure 1. Photomicrographs illustrating the progression from mammary hyperplasia to malignancy in the tpr-met transgenic mouse. (A) A fat-cleared, hematoxylin-stained mammary gland whole mount preparation showing large foci of mammary hyperplasia which stand out from the background as dark lobular structures (arrows) ($\times 17$). (B) A photomicrograph showing a focal hyperplastic alveolar nodule which stands out from the general background of mammary fat and ducts ($\times 88$). (C) A high magnification of a scirrhous carcinoma in a tpr-met transgenic mouse ($\times 220$). (D) A high magnification illustrating the papillary type of tumor from a tpr-met transgenic mouse ($\times 220$).

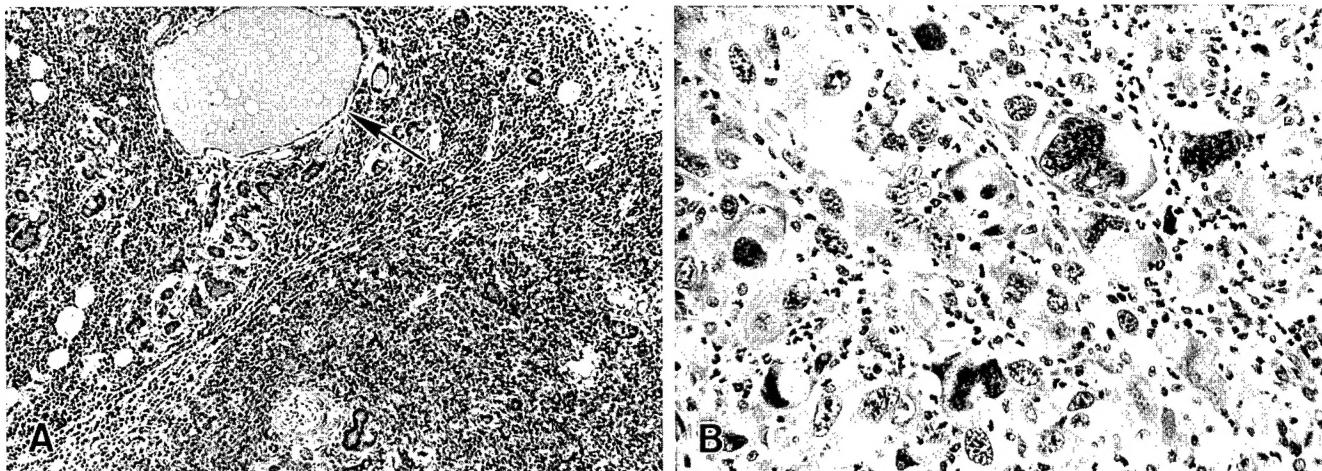


Figure 2. Photomicrographs illustrating two other tumors found in the tpr-met transgenic mouse. (A) A low magnification showing a small cell lymphoma which diffusely infiltrates a mammary fat pad leaving a cystically dilated duct (arrow) and several smaller mammary ducts (compare with 1b) ($\times 88$). (B) A high magnification photomicrograph illustrating a poorly differentiated giant cell tumor with osteoid formation ($\times 220$).

tional F1 females from this line, eight from line MTM2, and eight from line MTM3 were set up for forced continuous breeding (Table I). Most of the multiparous mice had mammary hyperplastic alveolar nodules (HAN) (Fig. 1, A and B) and several of them also had foci of microscopic carcinoma on whole mount examination. Seven primary mammary tumors developed in six of the MTM1 animals. In the MTM2 line, seven independent mammary tumors developed in three female animals. In the third line (MTM3), three mammary tumors were observed in two animals. The mammary tumors had one of three patterns, scirrhouss, papillary, or nodular (Fig. 1, C and D), many of them resembling human mammary tumors. The nuclei were intermediate in size with delicate clumped chromatin. Histological patterns of these types have not been seen in spontaneous murine tumors (21).

Additional tumors were also found in animals from all three lines (Table I). Single animals from MTM1 and MTM3 lines developed diffuse lymphoblastic lymphomas involving the mammary gland and lymph nodes (Fig. 2 A) at 6–9 mo of age. One MTM3 mouse developed a thymic lymphoma. Spindle cell sarcomas were also observed in MTM2 and MTM1 mice. An unusual orbital giant cell osteosarcoma was noted in another MTM1 animal at 18 mo of age.

Northern blot analysis was performed on total RNAs extracted from various tissues and breast tumors. Only the breast tumors contained detectable tpr-met transcripts in the expected size range (Fig. 3 A), whereas no signal was detected in any of the normal tissues examined (liver, stomach, intestine, breast, kidney, spleen). To improve the sensitivity of detecting low levels of the tpr-met transcript, RT-PCR was performed

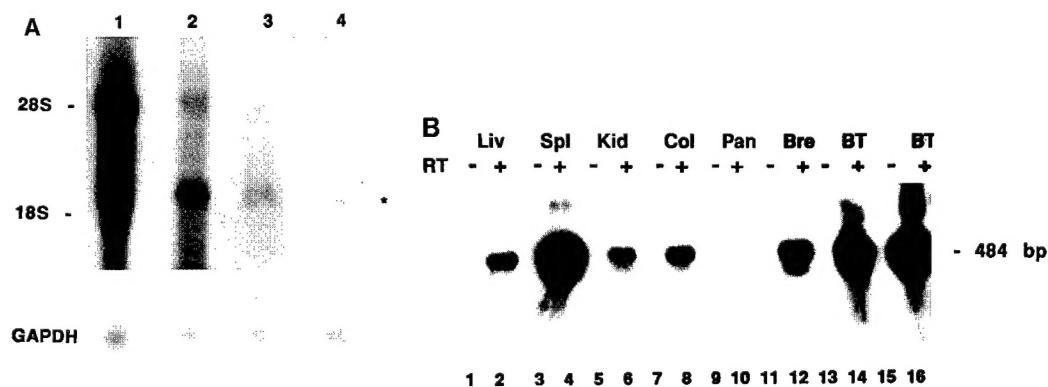


Figure 3. Analysis of tpr-met mRNA in MTM transgenic mice. (A) Expression of tpr-met mRNA in mammary tumors. RNAs were extracted and subjected to Northern blot analysis using a ^{32}P -labeled human c-met cDNA under stringent condition. An expected 2.5-kb RNA was detected. Some larger species of tpr-met RNA was also detected in some samples. Three mammary tumors (lanes 2, BT4; lane 3, BT3; lane 4, BT2) and one lymphoma (lane 1, Ly1) were analyzed in this blot. In contrast, tpr-met mRNA was not detected in normal tissues from both transgenic and nontransgenic litter mates (not shown). (B) Detection of tpr-met transcripts by reverse transcription-polymerase chain reaction (RT-PCR). Extracted RNAs from various tissues were subjected to RT-PCR as described (lanes as indicated). Control reactions without RT (−) were performed in parallel for each samples. Tissues shown include liver (Liv), spleen (Spl), kidney (Kid), colon (Col), pancreas (Pan), breast (Bre), and two breast tumors (BT2 and 3). 10 μg of RNA was used for RT-PCR of all samples except for the mammary tumors where 1 μg was used for the reaction.

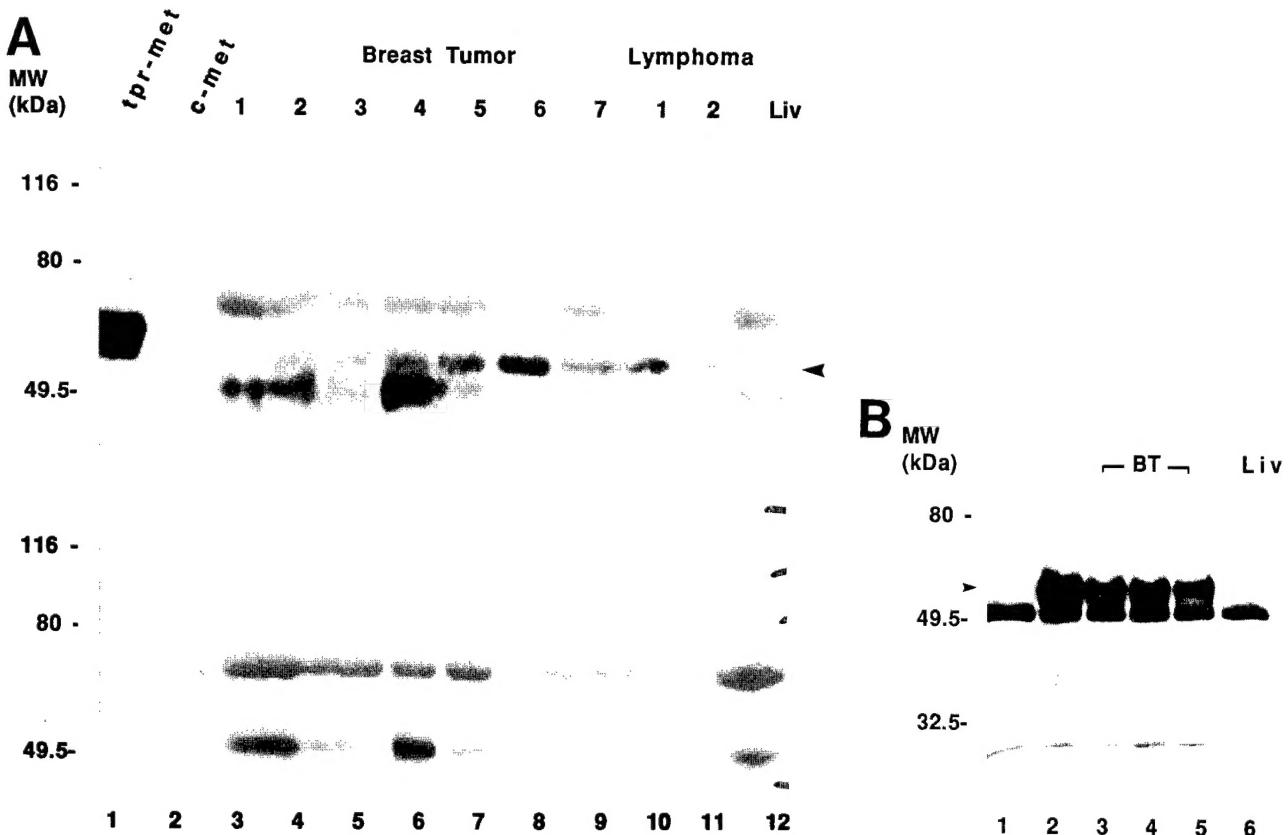


Figure 4. Analysis of tpr-met protein in tumors. (A) Tissues were homogenized using a Dounce homogenizer in the presence of RIPA buffer containing protease inhibitors. Cell lysates were cleared of nuclei and other cell debris by centrifugation, and 100 µg of lysates were subjected to Western blot analysis with anti-met 19S monoclonal antibody (from George Vande Woude) in 1:1000 dilution using a chemiluminescence kit (Amersham). A parallel control blot was set up using preimmune mouse serum. For positive controls (tpr-met and c-met), 20 µg of cell lysates from COS cells transfected with a tpr-met expression plasmid and HepG2 cells which expresses the 140-kD c-met protein were included. The lanes are as indicated and include data from seven breast tumors (BT1-7), two lymphomas (Ly1 and 2) and a transgenic liver. The top panel represents the blot probed with the 19S antibody and the bottom is the control. A specific 65-kD protein (arrowhead) was detected in most of the tumors and the tpr-met transfected cells. As expected, a 140-kD protein was detected in the HepG2 cells. (B) Analysis of tyrosine phosphorylation of tpr-met in tumors. 100 µg of Cell lysates (containing phosphatase inhibitors) from three breast tumors (BT5-7, lanes 3-5) and liver (lane 6) were immunoprecipitated with 19 S antibody, electrophoresed under reducing condition, and subjected to Western immunoblot with anti-phosphotyrosine antibody (Santa Cruz Biotechnology). As controls, 20 µg of cell lysates from untransfected COS cells (lane 1) and COS cells transfected with tpr-met expression construct (lane 2) were included. The 65-kD protein (arrowhead) was recognized specifically by the antibody since a parallel control blot using preimmune mouse serum was negative. The 50- and 25-kD bands detected on these blots are immunoglobulin heavy and light chains.

on RNAs extracted from these tissues. Using primers specific for the transgene, we were able to detect tpr-met transcripts in all the normal tissues (Fig. 3B). Although the primers were designed to span the SV40 intron, we detected PCR products of both unspliced and spliced RNA (differing by 66 nt) in all the samples. This observation, however, is consistent with the previous findings that SV40 intron is not particularly efficient in splicing (22). Western Immunoblot, performed on protein lysates extracted from various tumors, showed detectable expression of tpr-met protein in most tumor tissues (Fig. 4A). Analysis of normal tissues from all three lines of transgenic animals revealed no detectable tpr-met protein expression (not shown). In these tumors, the tpr-met protein also appeared to be phosphorylated on the tyrosine residue (Fig. 4B). This finding is consistent with the previous observation that tpr-met protein is constitutively phosphorylated and activated through dimerization (13).

Discussion

The pattern and occurrence of mammary hyperplasia and tumors in the tpr-met transgenic strains support strongly the conclusion that these lesions are a direct effect of the transgene. Hyperplasia and tumors developed in all three independent transgenic lines (MTM1, MTM2, and MTM3). In addition, 15 FVB/n multiparous mice were followed for the same length of time (average 1.5 yr) and did not develop any tumors. This observation is consistent with other investigators' experiences with the FVB/n strain (23). Furthermore, occurrence of multiple independent mammary tumors, as observed in some of our animals, is rare in spontaneous breast tumors of normal mice.

The use of a cellular promoter (metallothionein) rather than the MMTV promoter to drive the tpr-met transgene further speaks for the uniqueness of this animal model in the study of mammary carcinogenesis. The metallothionein pro-

moter enabled us to express low levels of tpr-met in a variety of tissues, including not only mammary epithelium but also liver, spleen, kidney, and colon. Our finding that MT-tpr-met transgene mice developed a predominant breast cancer phenotype might suggest that mouse mammary epithelium is susceptible to transformation by the tpr-met oncogene. In addition, it is interesting to note that only mammary tumors expressed high-level of the tpr-met transcript and protein, whereas normal tissues including mammary epithelium expressed low level. Perhaps, during physiological hyperplasia of mammary glands as a result of pregnancy, cells expressing higher levels of the oncoprotein were selected and clonally expanded to eventually form foci of mammary malignancy. This observation suggests that in this transgenic model, mammary adenocarcinoma developed as a direct effect of high-level expression of tpr-met. This is in contrast to some of the other transgenic models of breast cancer, in which basal expression of the transgene is high in normal epithelium (23). The reason for the relatively low level expression of the tpr-met transgene in all three lines is not clear. Since the metallothionein promoter is considered a relatively strong promoter and the transgene construct appears to work well in cell culture, the low RNA levels in various tissues could be due to instability and short half-life of the tpr-met transcript or to embryonic lethality of high level expression.

The c-met protooncogene product plays an important role in the growth and differentiation of epithelial cells in various organs. Recent studies have demonstrated a wide distribution of this protein in normal tissues, including breast, intestine, stomach, liver, pancreas, and kidney, and more interestingly, increased levels of met RNA and protein in a number of human tumors, particularly thyroid, gastric, hepatic, intestinal, and soft tissue tumors (15, 16, 24). It is interesting to note that several mesenchymal tumors (osteosarcoma, spindle cell sarcoma) were detected in the MTM mice. This observation is consistent with previous findings that tpr-met efficiently transform NIH-3T3 fibroblasts and that met overexpression occurs frequently in human sarcoma (8, 16). Examination of liver tissues in the MTM transgenic animals also revealed "nuclear unrest" of hepatocytes with increased mitotic activities (not shown). This observation is interesting in light of the potent mitogenic effect of HGF on hepatocytes (6).

A recent study showed that the c-met locus on chromosome 7 (7q21-22) was deleted in 41% of 245 patients with primary breast cancer (25). In addition, patients with loss of heterozygosity on chromosome 7q21-22 had significantly shorter metastasis-free survival and overall survival, suggesting that this region of chromosome 7, possibly the c-met gene, may be the site of a breast tumor or metastasis suppressor gene. Although our study suggests that tpr-met is a dominant oncogene in mammary carcinogenesis, it is possible that wild-type c-met may play a different role in growth and proliferation of mammary epithelium. Recent studies on the function of HGF-met activation suggested a potential regulatory role for c-met in the morphogenesis of mammary epithelium (26). The significance of genetic alterations involving c-met in human mammary carcinogenesis thus requires further study.

Co-expression of human c-met and its ligand HGF/SF has been shown to confer increased invasiveness and metastasis to NIH 3T3 cells in transfection studies (7). This phenomenon is probably mediated through an autocrine activation of c-met. In these experiments, transfection of the tpr-met oncogene

alone appears to be equally potent as the co-expression of met-HGF/SF, consistent with the observation that the tpr-met is constitutively activated. Furthermore, it is interesting to note that expression of tpr-met, but not of met-HGF/SF, appears to allow growth of transfected cells and tumor formation in heterogeneous immune competent mice (7). The precise mechanism by which tpr-met mediates this effect is unknown; it is possible that expression of tpr-met allows cells to escape immune surveillance. This intriguing idea underlies the possibility that tpr-met induces tumor formation in our transgenic mice through a mechanism independent of mitogenic effect of tpr-met. Further studies are necessary to resolve this issue. Although the tpr-met oncogene was originally generated by chemical mutagenesis in vitro and its existence has not been shown definitively in vivo, our study demonstrates that deregulated met expression can lead to development of mammary tumors in transgenic mice. This observation suggests that genetic alterations involving c-met may play an important role in the pathogenesis of human breast cancers. Furthermore, this transgenic line with high incidence of breast tumors can be a useful animal model for study of genetic and pathological alterations involved in the progression of mammary carcinogenesis.

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